Bioinformatic Analysis of lncRNA Mediated Competitive Endogenous RNA Network in Intestinal Ischemia/reperfusion Injury

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Research

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Abstract

Background

Recently, an increasing number of studies have reported the roles of competitive endogenous RNA (ceRNA) networks in ischemia/reperfusion (I/R) injury, which include the liver, kidney, heart, brain, and intestine. However, the functions and mechanisms of long non-coding RNAs (lncRNAs), which serve as ceRNA networks in intestinal I/R injury, are still unclear.

Methods

In this study, bioinformatics methods were used to filter and construct the lncRNA-miRNA-mRNA networks in intestinal I/R injury. RNA expression data were retrieved from NCBI GEO datasets, the expression profiles between mouse small intestine with superior mesenteric artery occlusion and Sham operation was analyzed, and 189 microRNA differential expressed genes(miDEGs) were discovered successfully from miRNA GEO dataset (GSE83701). Next, targeted lncRNAs and mRNA in the database were matched based on miDEGs. Then, lncRNA-miRNA-mRNA networks were constructed with Cytoscape. The hub lncRNA-miRNA-mRNA networks were selected via Cytoscape plug-in CytoHubba and intersected mRNAs of datasets GSE96733 and GSE83701.

Finally, the vital nodes of the ceRNA networks were validated by qPCR.

Results

The 1700020114Rik/mmu-miR-7a-5p/Klf4 axis was postulated to play a potential role in intestinal I/R injury.

Conclusion

The results shed novel insight into the molecular mechanism of ceRNA networks in intestinal I/R injury and highlighted the potential of 170002700020114Rik/mmu-miR-7a-5p/Klf4 ceRNA network in the prevention and treatment of intestinal I/R injury.

Introduction

I/R-induced organ injury is one of the leading causes of perioperative death worldwide[1]. Among the numerous I/R injuries, intestinal I/R injury is the most difficult to diagnose and treat. It could induce multi-organ function failure, even death, making it one of the major and demanding challenges in medical research. Intestinal I/R injury can result in subsequent aberrant regulation of oxidative stress apoptosis, mitochondrial dysfunction, autophagic pathways, and signal transducers activation[1]. However, the precise molecular mechanism of intestinal I/R injury is still unclear. In order to effectively reduce the damage of intestinal I/R injury and limit the multiple organ injuries caused by intestinal I/R injury, the

development of innovative treatment and in-depth study is urgently required. Non-coding RNA, including lncRNAs and microRNAs (miRNAs), have been shown to influence I/R injury recently[2].

LncRNA is a non-coding RNA with a length greater than 200 nucleotides which are not translated into a protein[3]. LncRNA has been shown to control gene expression by chromatin modification, transcriptional and post-transcriptional modifications[3]. MicroRNA(miRNA) is a small non-coding RNA with a length between 22–25 nucleotides, which can bind to target RNA transcripts resulting in the repression of mRNA expression or degradation of mRNA.

According to the ceRNA hypothesis proposed by Salmena et al. [4], lncRNAs and mRNAs may share miRNA response elements (MREs). There is growing evidence that lncRNA, mRNA, and circRNA could compete with miRNAs by sharing MREs[5], implying that lncRNAs operate as a sponge for miRNAs by binding with MREs, therefore suppressing the effect of miRNAs on their putative targets. Meanwhile, certain miRNAs can bind with the 3'UTR of lncRNAs or mRNA, causing degradation or suppressing the mRNA production.

The role of miRNAs in the I/R injury has been assessed in several tissues, including the intestine, liver, kidney, heart, and brain[2]. The molecular mechanism of I/R damage is expected to be elucidated by understanding lncRNA-miRNA interactions[2].

A growing number of studies have demonstrated the role of lncRNAs as a ceRNA in the occurrence and progression of I/R injury, including heart, liver, intestinal, and brain injury [5–7]. Xu et al.[7] found and validated that maladjusted lncRNAs were appeared in the early stage of the intestinal I/R injury and were associated with apoptosis. Feng et al.[8] elucidated that circle RNA PRKCB acts as a ceRNA and plays an essential role in intestinal I/R injury via modulating oxidative stress. However, the functions and mechanisms of most lncRNAs act as a ceRNA network in intestinal I/R injury are not well clarified. In this study, lncRNA mediated ceRNA networks related to the intestinal I/R injury were constructed by a set of analysis (Fig. 1).

Results

Differentially expressed miRNAs

GSE83701 yielded a total of 1193 miRNAs after preprocessing. A total of 189 miDEGs (107 up-regulated and 82 down-regulated) were obtained (Table 2). Principal component analysis (PCA) indicates that the sets of samples are independent of each other. The volcano plot in Fig. 2B demonstrates that there are two groups of differentially expressed miRNAs. The heatmap (Fig. 2C) revealed that differentially expressed miRNAs clearly separated the IR and Sham groups upon cluster analysis.

Prediction of mRNAs of miRNAs and construction of the network

The189 miDEGs were used to predict mRNAs in miRWalk 3.0.[9] Through filtering, 40 miRNAs (24 up-regulated,16 down-regulated) were found to be associated with 240 predicted target genes (Table 2). The network (Fig. 3A) based on these 40 miRNAs and 240 mRNAs were constructed and visualized by Cytoscape.

Gene Ontology Analyses of differentially expressed miRNAs

Figure 3B presents the results of the GO analysis for the miRNAs that the number of mRNAs is ≥ 3. The target genes(mRNAs) of downregulated mmu-miR-185-5p were mainly enriched in p53 binding, whereas the unregulated mRNAs of mmu-miR-7a-5p were significantly enriched in histone deacetylase binding, RNA polymerase II specific DNA-binding transcription factor binding, and DNA-binding transcription factor binding. The target genes of mmu-miR-106b-5p and mmu-miR-449a-5p were both enriched in repressing transcription binding. As shown in Fig. 3A,the two miRNAs were up-regulated. It is well known that miRNA can bind to target RNA transcripts, resulting in the repression of target gene expression. So, the function of transcription faction binding was activated, which corresponded to intestinal I/R injury.

Construction of PPI network

Based on the interactions retrieved from STRING, PPI network was constructed.

Between the 240 mRNAs, 60 mRNAs showed protein-protein interactions (Table 4), and 57 of them were interacted with 16 miRNAs in the above 40 miRNAs (Fig. 3A). In this network, most mRNA were targeted by mmu-miR-709, and there are interactions between its target genes.

CeRNA regulating network

The 40 miRNAs that matched mRNAs in the previous section were chosen to match lncRNAs to further highlight the link between lncRNAs and miRNAs. Upon a search, 26 miRNAs were found to be associated with lncRNAs, yielding 451 regulatory connections between miRNAs and lncRNAs (Table 5). MiRNA-lncRNAs that were validated by more than 3 CLIP-seq experiments(clipExpNum > 3)were included in the Starbase. Eight up-regulated, and 2 down-regulated miRNAs are matched with 8 lncRNAs following filtration (Table 6, Fig. 3C), and the resulting CeRNA regulatory network was built using miRNA-mRNAs (Fig. 3D). Then, Cytoscape was used to visualize the interacting relationships between mRNA, miRNA and lncRNA, which is shown in Fig. 4B. It is worth noting that mmu-miR-709 which were targeted by most mRNAs matched with none of the lncRNAs in starBase by now, so it was not included in ceRNA networks.

Validation of key genes in the ceRNA

The hub miDEGs (Fig. 3D) identified by CytoHubba were selected to reduce the scope of ceRNA networks. As shown in this ceRNA network, lncRNA 1700020I14Rik matched with miRNAs mmu-miR-363-3p and mmu-miR-7a-5p, while lncRNA Gm26917 matched with miRNAs mmu-miR-185-5p, mmu-miR-665-5p, and mmu-miR-339-5p. Then ceRNA networks were then used to derive four crucial lncRNA/miRNA/mRNA modules. GSE97633 dataset was applied to confirm the major genes in the ceRNA network of intestinal I/R injury depicted in the previous study. The same approach and criterion were employed to obtain the

mDEGs from GSE97633. Moreover, the mDEGs from GSE97633 were intersected with the anticipated mRNAs associated with miDEGs in dataset GSE83701. The results show that the 4 genes, which are Lrrc58, Sdad1, Klf4 and Cd69, are the intersection genes. The terminal results are: lncRNA 1700020114Rik/mmu-miR-7a-5p/Klf4 and lncRNA Gm26917/mmu-miR-665-3p/ Lrrc58 network (Fig. 3D).

Real-time Polymerase Chain Reaction (RT-PCR)

H&E staining and histological injury scoring (Chiu's score) of the intestinal mucosa were shown in Fig. 4A-C. The qRT-PCR was used to measure the expression of above ceRNAs. The expression of lncRNA 1700020114Rik and Klf4 decreased markedly ($p < 0.05$) in intestinal I/R injury, while the mmu-miR-7a-5p and Lrrc58 expression level increased, as shown in Fig. 4D-F and I. There was no statistical difference in the expression of lncRNA Gm26917 and miRNA mmu-miR-665-5p between the two groups (Fig. 4G-H). Finally, lncRNA 1700020114Rik/mmu-miR-7a-5p/Klf4 ceRNA network was constructed.

Discussion

LncRNA, regulates the expression of protein-coding genes via functioning as miRNA sponges. Numerous studies have elucidated that lncRNAs extensively participate in crucial physiological processes such as metabolism and immunity, and are closely linked to the emergence and progression of tumors, cardiovascular diseases, nervous system disorders, nephropathy, and other diseases[10]. However, the mechanism of lncRNA-mediated ceRNA in intestinal I/R injury remains unclear. In this study, the expression datasets of miRNA of intestinal I/R injury samples were collected from the NCBI GEO datasets GSE83701. LncRNA and mRNA were predicted by database through miDEGs, and lncRNA 1700020114Rik/mmu-miR-7a-5p/Klf4 ceRNA network was constructed by Cytoscape. The animal experiments validated that LncRNA 1700020I14Rik, Klf4 were downregulated. Meanwhile, miR-7a-5p was up-regulated in intestinal I/R injury. Besides,there has been no report of the 11700020114Rik/mmu-miR-7a-5p/Klf4 ceRNA network in intestinal I/R injury so far.

LncRNA1700020I14Rik is also known as Oip5os1 and Oip5as1.Studies[11, 12] have elucidated that lncRNAs 1700020I14Rik play a key role in apoptosis, cell proliferation and fibrosis, oxidative stress, and other physiological processes. In addition, other studies[11, 13] revealed that LncRNA 1700020I14Rik was downregulated in the hearts of rats with myocardial I/R injury and hypoxia / reoxygenation(H/R)injury in myocardial cells. Researchers discovered that sponging miR-186-5p with up-regulated lncRNA OIP5-AS1 reduced neuron damage in microglia/macrophages following middle cerebral artery occlusion/reperfusion produced inflammation and oxidative stress.[14]

The mmu-miR-7a-5p sequence in Mus musculus is a product of miR-7 genes. miR-7a-5p has controversial roles in multi-organ injury. Some researches[15, 16] found that miR-7a-5p may play a protective role in intestinal and brain. However, other studies demonstrated that miR-7a-5p was up-regulated during the cerebral I/R injury[17], acute lung injury[18], and myocardial injury induced by lipopolysaccharide [19]. The qRT-PCR results showed that miR-7a-5p was up-regulated after intestinal I/R injury.

Klf4 can be regulated by miRNA at both transcriptional and post-transcriptional levels[20]. Klf4 is a multi-functional transcription factor that regulates diverse cellular processes in a context-dependent manner[20]. Thus, the role of Klf4 is determined by different genes interacting with Klf4. Hummitzsch et al.[21] found that the expression level of Klf4 was decreased in Human Regulatory Macrophages after H/R. Meanwhile, Klf4 could alleviate lipopolysaccharide-induced inflammation and cerebral vascular injury after cerebral ischemic stroke [22, 23]. Therefore, Klf4 could help to alleviate I/R injury.

Overall, the lncRNA 1700020I14Rik, Klf4 and miR-7a-5p were associated with ischemia/reperfusion injury or oxygen-glucose deprivation injury.

In addition, lncRNA 1700020I14Rik and Klf4 were downregulated, and miR-7a-5p was up-regulated in ischemia/reperfusion injury in the ceRNA network identified by the bioinformatics analysis. This was in accordance with the experimental validation in intestinal I/R injury. Furthermore, studies[18] have confirmed that miR-7a-5p has interactions with Klf4 in acute lung injury.

Therefore, the 1700020114Rik/mmu-miR-7a-5p/Klf4 axis was postulated to play a potential role in intestinal I/R injury. It was the first time to uncover lncRNA mediated ceRNA networks in intestinal I/R injury. However, the role of 1700020114Rik/mmu-miR-7a-5p/Klf4 ceRNA network in intestinal I/R injury needs to be examined in further studies.

Conclusion

The findings provide a fresh insight at ceRNA regulation mechanism in intestinal I/R injury, identifying 170002700020114Rik/mmu-miR-7a-5p/Klf4 ceRNA network as potential targets for prevention and treatment of intestinal I/R injury.

Materials And Methods

MiRNA and mRNA Microarray Data

The GEO database, which is a gene expression database created and maintained by the National Center for Biotechnology Information (NCBI), stores curated gene expression datasets. The miRNA/mRNA expression profiles GSE96733[24] and GSE83701[8] from NCBI-GEO were used in mice intestinal I/R injury study.

Differentially expressed miRNAs analysis

The data was divided into two groups: the IR group and the Sham group. The limma package (Version 3.26.9)[25] and Bayes test were employed to identify the differentially expressed miRNAs/mRNAs (miDEGs and mDEGs) between the sham samples and intestinal I/R injury samples. Multiple-test correction with Benjamini and Hochberg's method was performed for *p*-value calculation. Genes with *p*-value < 0.05 and |log2FC| > 1.5 were regarded as the thresholds of miDEGs or mDEGs. Gene with *p*-value

< 0.05 and log2FC > 1.5 are defined as up-regulated miDEGs/mDEGs, and with *p*-value < 0.05 and log2FC<-1.5 are defined as down-regulated miDEGs/mDEGs. The pheatmap package (version 1.0.12)[26] was applied for the bidirectional hierarchical clustering, and the expression values were presented using heatmaps.

Predicting miRNA-mRNAs

Their potential mRNAs that matched with miDEGs were predicted by miRWalk 3.0 [9]. In addition, target mRNAs were validated by experiments and simultaneously predicated by the miRDB or Targetscan databases.

Protein-protein interaction(PPI) network generation and module analysis

The STRING database [27] (version:11.0b,https://string-db.org)

was used to construct PPI network for target genes-encoded proteins, including direct and indirect associations. The Required Confidence (combined score) > 0.7 [27] was set as threshold value. The Cytoscape was utilized to visualize the raw data of network from STRING database and then displayed [28](version 3.8.0, https://www.cytoscape.org). MiRNA nodes of every gene were added to PPI network to analyze the main regulatory mechanism of miRNAs.

Gene Ontology Analyses of differentially expressed miRNAs

Gene Ontology (GO) enrichment analysis was constructed as functional analysis of miDEG-targeted mRNA. The GO analysis was performed in R software with clusterProfiler package[29] and was visualized by ggplot2 package[30] in R Studio.

Construction of ceRNA regulating network

lncRNAs that target MiRNAs were searched using starBase Version [31] (Version 3.0,http://starbase.sysu.edu.cn)

also called ENCORI. The interacting relationships were visualized via Cytoscape. The lncRNA-miRNA network and miRNA-mRNA genes network were combined to form lncRNA-miRNA–mRNA network, also known as the ceRNA network[32]. The hub lncRNA-miRNA–mRNA networks were selected by Cytoscape plug-in CytoHubba[33]. Finally, the critical ceRNA network of intestinal I/R injury was selected using mRNAs found in both GSE97633 mDEGs and the target mRNAs of GSE83701 miDEGs.

Confirmation of RNA expression of ceRNA after intestinal ischemia/reperfusion injury

Animal Model

Male C57BL/6(weighing 20-25g, 7-8w) mice were obtained from Nanfang Hospital, Southern Medical University, and then housed at separated cages in a temperature-controlled room under a fixed circadian rhythm with free access to food and water. The animal experiments were approved by the Ethics Committee of Nanfang Hospital (China, application No: NFYY-2021-54)and all procedures were carried out in compliance with National Institutes of Health guidelines for the use of experimental animals. According to a previous study, the intestinal I/R injury was generated by occluding the superior mesenteric artery (SMA) with a microvascular clamp for 45 mins followed by 240 mins of reperfusion. In addition, the Sham group was the same with intestinal I/R injury except occluding the SMA [8],[34].

Sample Collection and Histological Staining

Intestine segments were collected at the end of reperfusion and then divided into two segments, based on past research [8]. One of the segments was dried with filter paper and preserved at -80°C, waiting for detection. The other was treated in 4% paraformaldehyde as previously described [35]. The intestinal injury score were measured by automated image analysis in five random 200X fields of each sample [36].

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) analysis

Trizol reagent was used to extract total RNA according to the manufacturer's protocol. The mRNA and miRNA was reversed into cDNA by kits from Toyobo and Takara, according to the previous study[36].LncRNA and mRNA quantitative analysis was conducted with qRT-PCR using Toyobo SYBR Green Realtime PCR (Applied Biosystems, Foster City, CA),whereas the expression of miRNA was evaluated via Takara TB Green Advantage qPCR Premix (200rxn, Cat.No.639676) .18S and U6 served as internal controls, and the fold change relative to the control was computed by the $2^{-\Delta\Delta Ct}$ method. Table 1 displyas the Forward and Reverse primer sequences.

Statistical Analysis

Statistical analyses were conducted using R (version 4.0.3) and GraphPad Prism version 8.0 (GraphPad Software, Inc., San Diego, CA, USA).

The results were provided as mean ± standard error of the mean (SEM) from at least three independent replicates. Welch's t-test was used to estimate the differences between the groups. The statistically significant difference was defined as a *P* value less than 0.05.

Declarations

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Author Contributions

Lin Zhu, Xiao Yang, Kexuan Liu and Bingcheng Zhao designed experiments, analyzed data and prepared this manuscript. Ziyi Wang,Yupei Lai and Shiting Xu performed animal experiments, ZhiWen Yao and Lin Zhu discussed the results, Kexuan Liu and Bingcheng Zhao revised this paper. All authors reviewed and approved the submitted the manuscript.

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Availability of data and materials

Data could be obtained upon request to the corresponding author.

Ethics approval and consent to participate

The animal experiments were approved by the Ethics Committee of Nanfang Hospital (China, application No: NFYY-2021-54). There are no clinical experiments in this study.

Consent for publication

Not applicable

Conflict of interest

The authors declare that they have no competing interests.

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Tables

Due to technical limitations, tables are only available as a download in the Supplemental Files section.

Figures

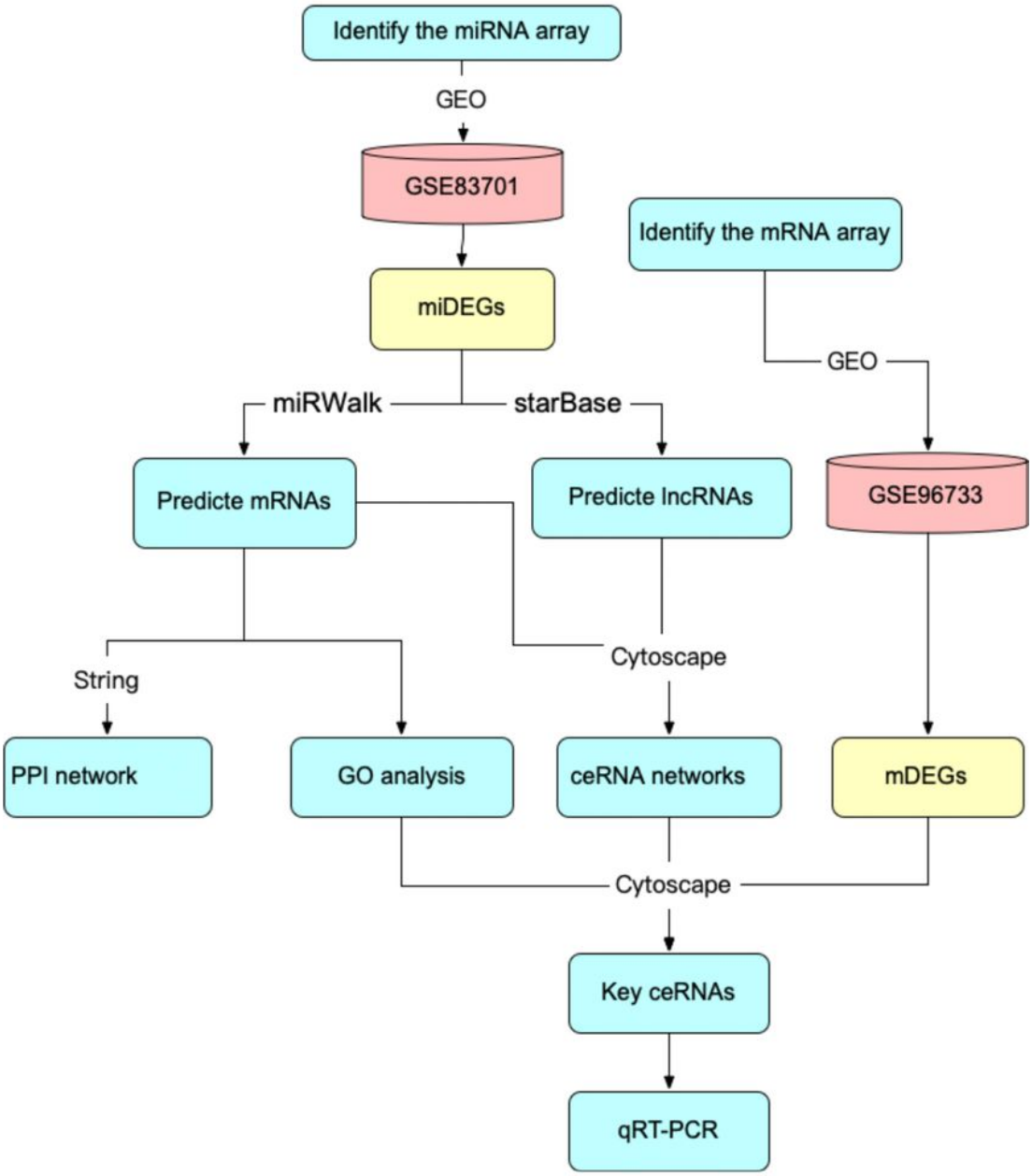

Figure 1

The flow chart of the study.

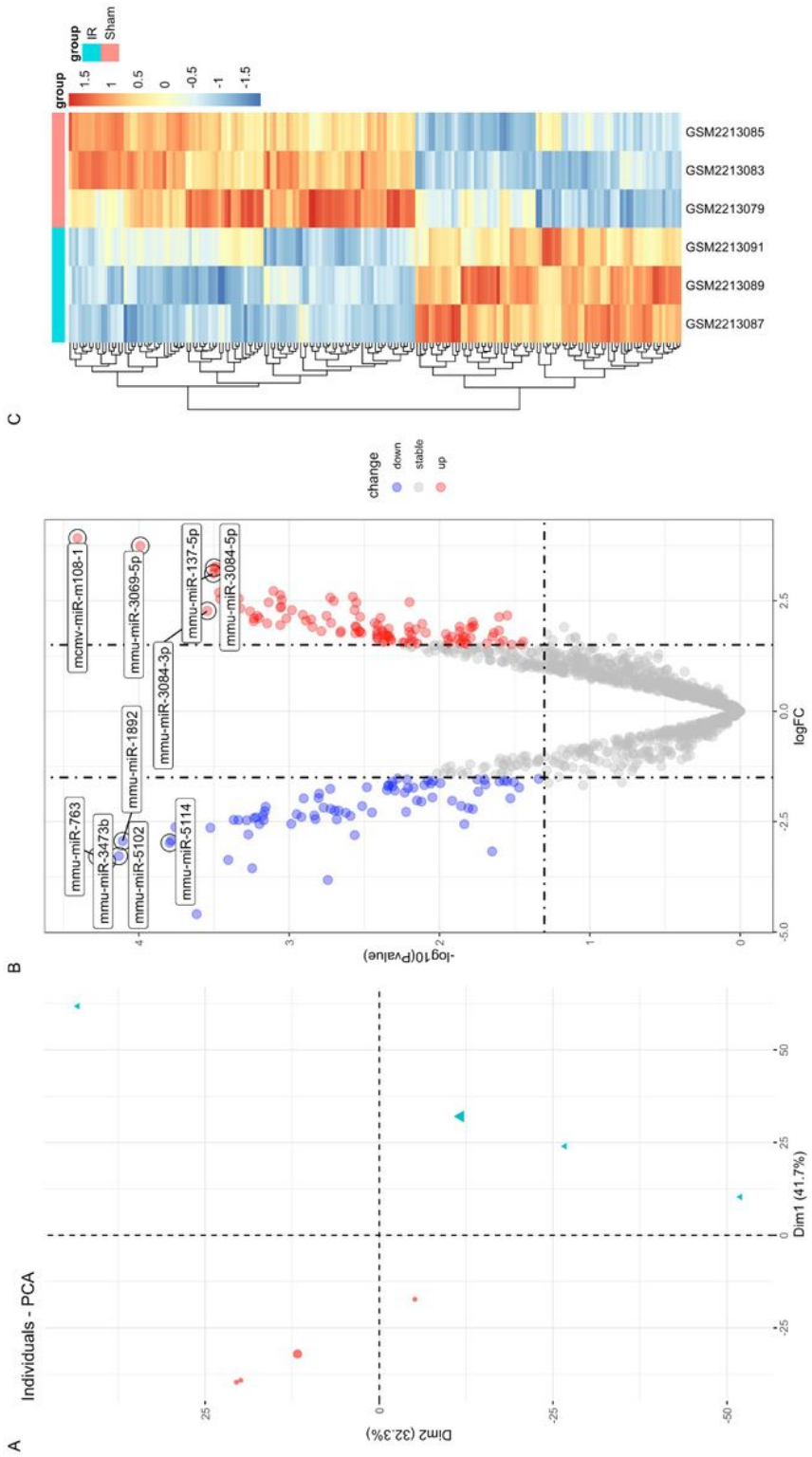

Figure 2

Differentially expressed miRNAs: (A) The PCA plot. (B) In the volcano plot of miDEGs, the horizontal axis represents the fold change (IR versus Sham), and the vertical axis represents the p-value of miDEGs, genes with the absolute value of |log2FC |>1.5 and p-value. (C) In the heat map of miDEGs, the horizontal axis represents the name of each sample, while the left vertical axis represents the degree of gene clustering. Red represented the up-regulated genes, while blue stands for the downregulated genes.

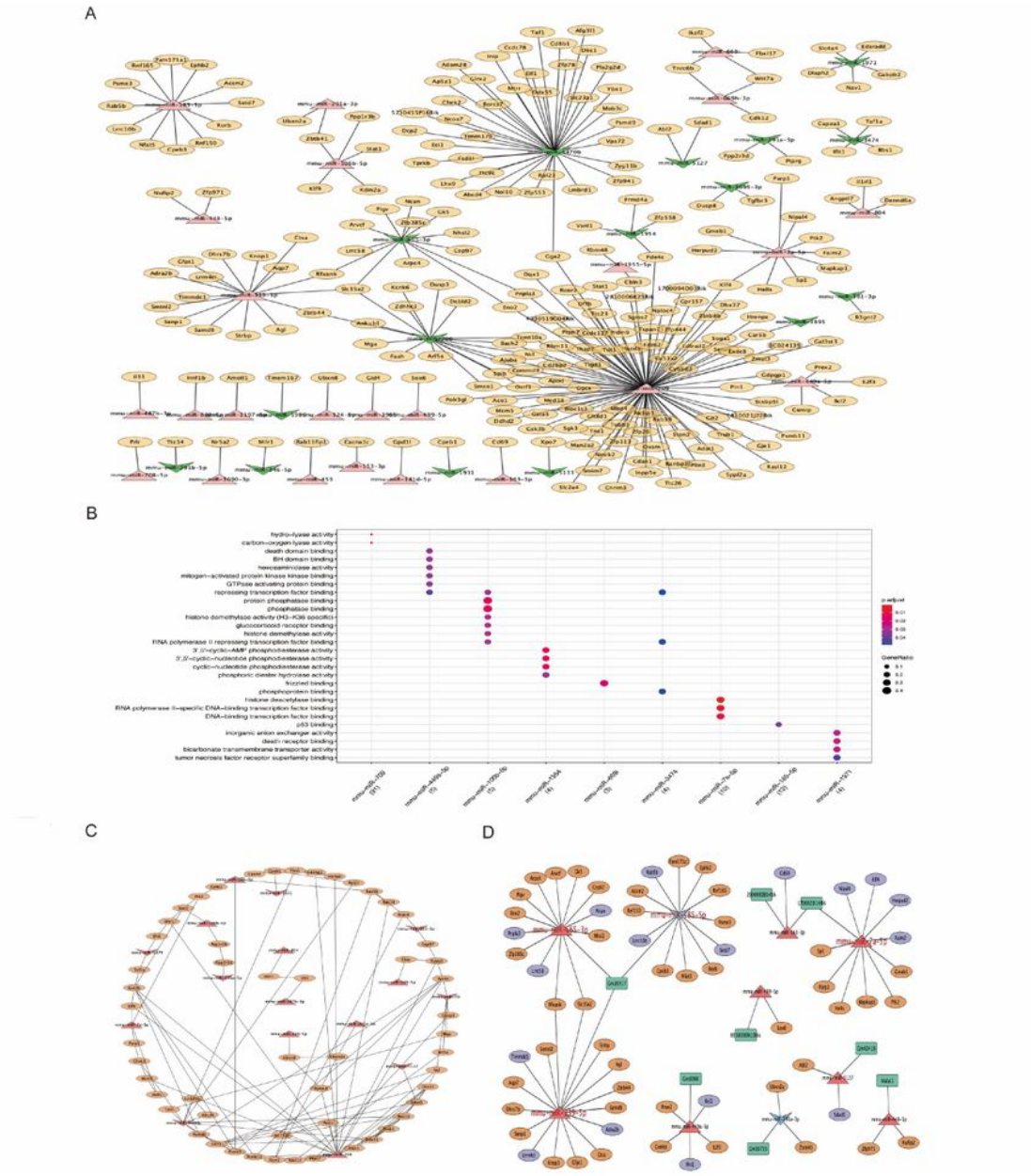

Figure 3

The miRNA-mRNA network, GO analysis, PPI and ceRNA networks. (A)The miRNA-mRNA network. The network was based on these 40 miRNAs and 240 target genes. Yellow circle nodes: target genes; red triangle: up-regulated miRNAs; grey arrow: down-regulated miRNAs. (B)The result of GO analyses for the miRNAs. The lateral axis: miRNAs, numbers in parentheses: the number of genes; vertical axis: the name of GO terms; the size of round dot: GeneRatio, the bigger dot represents more miRNAs enriched in this

term, the smaller p-value represents higher significance. (C)Protein-protein interaction (PPI)networks. Orange circle nodes: mRNAs; red triangle: up-regulated miRNAs; green arrow: down-regulated miRNAs; line without arrow: PPI relations; line with an arrow: miRNA-gene regulating relations. (D)Competing endogenous RNAs (ceRNA) regulating networks. Yellow circle nodes: target mRNAs；purple circle nodes: intersected target mRNAs between two datasets; red cycle: lncRNA; green arrow: down-regulated miRNAs; red triangle: up-regulated miRNAs, miRNA with red label were hub miRNAs chosen by cytoHubba.

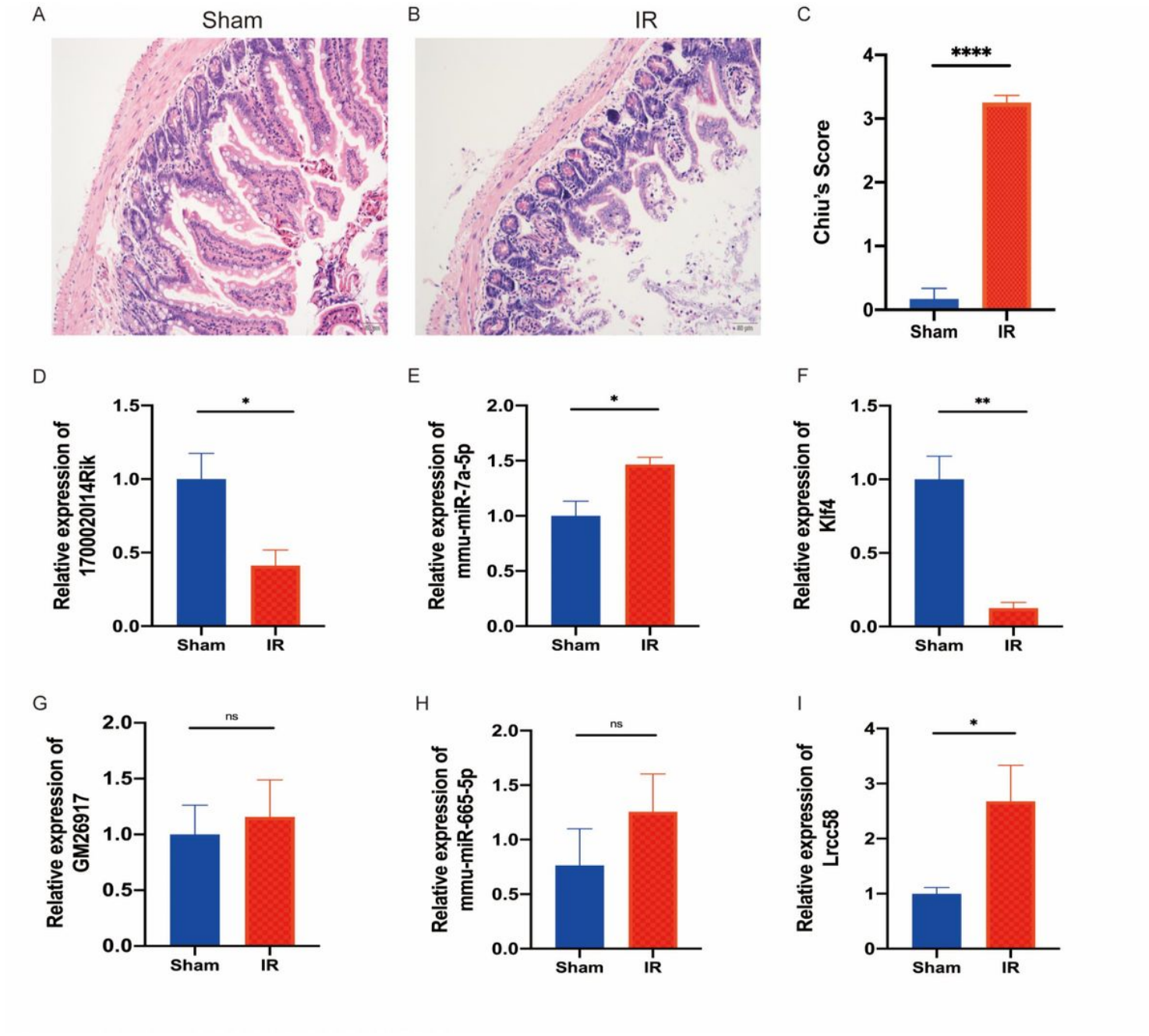

Figure 4

Histological injury scoring and qRT-PCR results of the small intestine. (A-B) H&E staining and histological injury scoring (Chiu's score) of the intestinal mucosa. Scale bar, 50μm(n=5,6). (C-H) Relative expression

of the lncRNA/miRNA/mRNA by qRT-PCR (n=4-6). The results are expressed as the mean ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001 ****p < 0.001 by two-tailed Welch's t-test.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ZhaoSupplymentaryFigure1.docx
- ZhaoSupplymentaryTables.xlsx